iPS cell therapy for Parkinson's disease

Peng, Suping

CHAPTER 4

POTENTIAL ROLE OF CELL ADHESION MOLECULES IN THE NEURITE OUTGROWTH OF IPS CELL-DERIVED DOPAMINERGIC NEURONS

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Submited

ABSTRACT

Intrastriatal transplantation of dopaminergic neurons has been shown a potentially very effective therapeutical approach for the treatment of Parkinson's disease (PD). With the detection of induced pluripotent stem cells (iPS cells), an unlimited source of autologous dopaminergic (DA) neurons became available. Although the iPS cell-derived dopaminergic neurons exhibited most of the fundamental dopaminergic characteristics, they showed some aberrations in the expression of genes involved in neuronal development and neurite outgrowth. The limited outgrowth of the iPS cell-derived DA neurons may hamper their potential application in cell transplantation therapy for PD. In the present study, we examined whether the forced expression of L1CAM and PSA-NCAM, via gene transduction, promoted the neurite formation and outgrowth of iPS cell-derived DA neurons. In cultures on astrocyte layers, both adhesion factors significantly increased neurite formation of the adhesion factor over-expressing iPS cell-derived DA neurons in comparison to control iPS cell-derived DA neurons. However, when plated on postnatal organotypic striatal slices that already intrinsically expressed high levels of L1CAM and PSA-NCAM, no significant differences in maximal neurite formation between the control and the adhesion factor over-expressing iPS cell-derived DA neurons could be detected. We examined the neurite outgrowth of the L1CAM- or PSA-NCAM-over-expressing iPS cell-derived DA neurons after implantation in the striatum of unilaterally 6-OHDA-lesioned rats; unfortunately, only a small number of the sorted and virally transduced cells survived the stressful implantation procedure. No apparent increased neurite outgrowth of these surviving DA neurons was observed presumably due to their reduced viability and health, annihilating the potential growth promoting effects of the adhesion factors.

INTRODUCTION

The specific and progressive loss of dopaminergic (DA) neurons in the substantia nigra (SN) is a major hallmark of Parkinson's disease (PD) [1]. At the time point that patients are diagnosed with PD, the loss of SN DA neurons is usually already more than 60%, resulting in a more than 80% depletion of dopamine in the striatum. Neither DA neuron loss nor striatum denervation is reversible. However, intrastriatal transplantation of DA neurons and the subsequent restoration of the striatal dopamine level have proven to be an effective symptom-reducing treatment for PD [2-3].

Nowadays, induced pluripotent stem cell (iPSC)-derived DA neurons are considered the most promising source for DA neuron grafts for PD patients. iPS cells are obtained by reprogramming of somatic cells such as skin fibroblasts [4-5], which can be collected from the PD patient via a small skin biopt; the autologous origin of the iPS cell-derived DA neuron graft makes immunosuppression superfluous. Since iPS cells have an unrestricted capacity for self-renewal, a principally unlimited number of cells can be obtained for transplantation purposes. Various strategies have been developed to differentiate the pluripotent cells towards a ventral mesencephalic (VM) DA neuron fate [6-8]. DA neurons derived from iPS cells show properties resembling primary VM DA neurons, such as the expression of specific markers LMX1a, NURR1, TH, PITX3, GIRK2 [9], the spontaneous or stimulated release of dopamine and the spontaneous action potential firing with a slow pace (1-10 Hz) [10-11]. However, when grafted into the striatum, the neurites from the iPS cell-derived DA neurons appear to be restricted within the injection tracks [9] or only shortly extending from the site of the graft [11].

To achieve significant functional improvement, a substantial reinnervation of the affected putamen and caudate is essential. In contrast to pluripotent stem cell-derived DA neurons, primary foetal VM DA neurons have been shown to be able to extensively reinnervate the grafted striatum [12], providing the proof-of-principle of the beneficial functionality of DA neuron grafts in PD patients [2, 13]. Even when 10 times more TH+ cells were implanted, pluripotent stem cell-derived DA neurons gave rise to a much lesser extent of striatal reinnervation than primary foetal VM DA neurons [14].

An approach to improve and promote the outgrowth of implanted iPS cell-derived DA neurons could be the forced over-expression of cell adhesion molecules that are rich in the developing foetal brain, in particular L1CAM and PSA-NCAM. L1CAM and PSA-NCAM are crucial cell adhesion molecules for embryonic neuron development and axon guidance. During the foetal development of VM DA neurons, the PSA-NCAM content increases strikingly in both the developing striatum and the mesencephalon, with TH+ cells clearly outlined by PSA-NCAM [15]. L1CAM is also expressed in foetal VM DA neurons and particularly mostly enriched in the TH+ fibers in the medial forebrain bundle (MFB) [16]. It has been demonstrated that specific stimulation of the L1CAM signaling pathway in VM tissue grafts by L1 Ab (Neomarkers, Lab Vision, Fremont, CA) resulted in a significantly greater area of graft-derived innervation compared to control grafts [17].

In the present study, we aimed to examine the effect of L1CAM or PSA-NCAM (by transduction of the gene encoding for STX, the enzyme that adds PSA onto NCAM) over-expression on the neurite outgrowth of mouse iPS cell-derived DA neurons *in-vitro* as well as *in-vivo* after transplantation in the Parkinson rat model. By generating iPS cells from the fibroblasts of Pitx3$^{gfp/+}$ transgenic mouse, we were able to get a pure population of DA neurons after their specific differentiation by FAC-sorting.

MATERIALS AND METHODS

iPS cell culture and DA neuron differentiation

Mouse Pitx3$^{gfp/+}$ iPSC clones CI, CII, CIII (passage 15–52) were maintained on γ-irradiated mouse embryonic fibroblasts in ES medium (Knockout DMEM, 15% knockout serum replacement, 1% nonessential amino acids (all Invitrogen, Breda, The Netherlands, www.invitrogen.com), 2 mM L-Glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (all PAA, Germany, www.paa.com), 100 µM β-mercaptoethanol) supplemented with 1000 U/mL Leukemia inhibitory factor (LIF; Millipore, Amsterdam, The Netherlands, www.millipore.com).

The DA differentiation protocol for mouse iPS cells was based on the MS5 protocol [18]. iPSC colonies were manually dissected and plated on MS5 cells in KSR medium (DMEM, 15% knockout serum replacement, 100 µM β-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin) from day 2–5. SHH (200 ng/ml, Peprotech), FGF8 (100 ng/ml, Peprotech), were added to the medium from day 6–8 of differentiation. Medium was changed into N2 medium (DMEM/F12 (Invitrogen), 2% N2 supplement (PAA), 2 mM L-Glutamine, penicillin/streptomycin, 1% sodium pyruvate (Invitrogen)) supplemented with SHH, FGF8, combined with FGF2 (100 ng/ml, Peprotech) from day 9-11. Followed by a final differentiation step in the presence of BDNF (20 ng/ml, Peprotech), GDNF (20 ng/ml, Peprotech), TGFβ3 (1 ng/ml, Peprotech), ascorbic acid (200 µM) and dbcAMP (100 µM) until the appearance of dopaminergic neuronal cell types.

RT-PCR and q-PCR

Total RNA was extracted from transduced iPS cells or differentiated iPS cells using standard RNA easy kit (Qiagen). Primers were used for L1, STX, Nestin, Pax6, Map2, β-III-tubulin, Nurr1, Pitx3, TH, and DAT. Primer sequence information is presented in Supplementary Table 1. Relative gene expression levels were determined by qPCR real-time pcr (Lightcycler) using the QuantiTect™ SYBR® Green PCR (QIAGEN) LightCycler® kit (Roche, Idaho Technologies) according to the manufacturer's instructions. For each reaction 0.1 ng total RNA was used as input. Relative expression levels were normalized to housekeeping gene HMBS.

Quantitatification of dopamine release by LC-MS/MS

The dopamine production by the iPS cell-derived DA neurons was analyzed as described earlier [19]. We analyzed 100 µl of supernatant from terminally DA-differentiated iPS cells under stimulation by 56 mM KCl.

Immunostaining

Cell cultures of iPS cell-derived cells were fixed with 4% paraformaldehyde for 15 minutes and washed 3 times with PBS before staining. Antigen retrieval was performed on brain cryosections with 10 mM Na citrate solution for 10 min heated in a microwave. Immunostaining was performed for the following markers: rabbit anti-tyrosine hydroxylase (1:500, AB152, Chemicon), mouse anti-GFP (1:500, MAB3580, Millipore), mouse anti-L1CAM (1:400, ab24345, Abcam), mouse anti-PSA-NCAM, IgM (1:400, MAB5324, Millipore). Samples were incubated for 24 hrs with primary antibodies at 4°C and primary antibodies were detected with appropriate secondary antibodies, coupled to Alexa 594 or Alexa 488 (1:800, Jackson ImmunoResearch, West Grove, USA) for 2–3 hrs at room temperature. Samples were counterstained for 10 min with Hoechst to visualize cell nuclei.

Western blotting

SDS-PAGE gel electrophoresis and Western Blot analysis were performed to detect Nanog, Oct3/4, Sox2, UTF1 and L1CAM expression in iPS cells. The following antibodies were used: rabbit anti-Nanog antibody (1:1000, ab80892, Abcam, Cambridge, UK), mouse anti-Oct3/4 antibody (1:500, sc-5279, Santa Cruz), rabbit anti-Sox2 (1:4000, ab15830, Abcam, Cambridge, UK), rabbit anti-UTF1 (1:2000), mouse anti-L1CAM (1:1000, ab24345, Abcam) and mouse anti-β-Actin (ab6276, Abcam, Cambridge, UK) at 1:10,000 dilution. The housekeeping gene *β-actin* has been used as loading control. Primary antibodies were detected using fluorescently labeled secondary antibody: donkey anti-mouse (IRDye® 680, LI-COR, Biosciences) and donkey anti-rabbit (IRDye® 800CW, LI-COR, Biosciences) according to manufactures instructions.

FACS

Differentiated iPS cell-derived cells were dissociated using the Papain Dissociation Kit (Worthington Biochem, Products, LK003150). Dissociated cells were collected in colorless DMEM (Gibco) with BDNF, ascorbic acid, GDNF, TGFβ3 and dbcAMP (see above), and sorted on a MoFlo-XDP or MoFlo-Astrios sorter with a 100 µm nozzle at a pressure of 15-17 psi based on GFP expression. Cells were collected in N2 medium with BDNF, ascorbic acid, GDNF, TGFβ3 and dbcAMP. Cells were spun down at 300 rcf for 10 min and resuspended in PBS.

Gene transduction

Sorted GFP+ iPS cell-derived DA neurons were transduced with lentiviral vectors containing genes encoding for human L1CAM, human STX (ST8Sia II) under the EF-1a promoter; an empty vector was used as control.

Lentiviral particles were produced by co-transfecting HEK 293T cells (Invitrogen, Carlsbad, CA) with the lentiviral vector pCMV Δ8.91 and vesicular stomatitis virus G protein (VSV-G) plasmid using Fugene (Fugene-HD; Roche, Almere, The Netherlands) in a six-well plate following the procedure provided by the manufacturer. The ratio used was 3 µl of Fugene to 1 µg of DNA for each well. After overnight transfection, culture medium was changed to N2 medium; 24 hrs later, media containing virus and 4 µg/ml polybrene (Sigma-Aldrich, Zwijndrecht, The Netherlands) were mixed and filtered through a 0.45-µm filter (Whatman, The Netherlands). The viral particles were further concentrated 100-fold by ultracentrifugation (20,000 g, 20 min). The pellet was resuspended in N2 medium.

For transduction, N2 medium containing lentiviral particles was added to the resuspended sorted cells in a prelubricant EP tube. After 4 hrs transduction, cells were washed twice by adding N2 medium, spun down at 300 rcf for 10 min and resuspended in PBS for grafting in 6-OHDA rats or in N2 medium for culturing on an astrocyte layer (4,000 cells/well) or on organotypic striatal slices (400 cells per slice). After 1 week of culturing, samples were taken for qPCR analysis or for immunocytochemistry staining.

Culture of primary astrocytes

Brain tissue was explanted from P1-P3 mice, meninges were peeled off and the brain tissue was chopped into small pieces and incubated with TE at 37 ◦C for 30 minutes. Glia medium (DMEM, with 10% FBS, 1% sodium pyruvate (Invitrogen), 100 U/mL Pen/Strep) was added to stop the digestion and tissue was pipetted into cell suspension and cultured at 37 ◦C, 5% CO_2. Medium was changed every 3-4 days. The flasks of cell culture were shaken at 150 rpm, 37 ◦C for 1 hr, for 3 times every other day to remove microglia. The remaining astrocytes were passaged into a 24 well culture plate as monolayer for sorted iPS cell-derived DA neurons.

Culture of organotypic striatal slices

Organotypic striatal slice cultures were performed with a modification of a previously published method [20]. Striata were dissected from P1-P3 mice and cut on a vibratome coronally at 250 mm and collected in ice-cold F12 medium. Slices were cultured at the liquid–air interface on Millicell CM culture inserts and maintained in Neurobasal medium supplemented with 0.5% B27 supplement, 2 mM l-glutamine, 25% horse serum, 25 mg/ml gentamycin, at 35 ◦C, 5% CO_2. Medium was changed every other day.

Neurite tracing and analysis

Neurite tracing was performed with 'simple neurite tracer' in Fiji (http://fiji.sc/Simple_Neurite_Tracer). The total neurite length represented the sum of the lengths of all neurites of one TH+ neuron. The number of primary neurites per neuron represented the number of neurites directly extending out from a TH+ soma. The number of branches per neuron comprised the total number of neurites traced minus the number of primary neurites. The effective area comprised the area of the polygon created by linking up the far ends of all neurites of one neuron using the polygon selection option in Fiji.

Lesions, rotation behaviour and transplantation surgery

Adult female Sprague Dawley (Harlan) rats (180–230 g) were housed under standard conditions with free access to food and water. Rats were anaesthetized with ketamine (90 mg/kg) and xylazine (4 mg/kg). Unilateral retrograde destruction of dopaminergic neurons in the substantia nigra was induced by 2 stereotaxic injections of 2.5 µl 6-OHDA (3 mg/ml in 0.2% ascorbic acid and 0.9% saline, Sigma) in the medial forebrain bundle of the nigro-striatal pathway (stereotaxic coordinates 1: AP -4.0, ML −0.8 and V −8.0; toothbar set at +3.4. and coordinates 2: AP -4.4, ML −1.2 and V −7.8; toothbar set at −2.4). The unilateral destruction of the nigro-striatal pathway was confirmed by the recording of d-amphetamine (5 mg/kg)-induced rotation behavior 2 weeks post lesion. Amphetamine-induced rotation scores were obtained using Rota-8 software (UMCG). Rats that rotated more than 5 rpm were included. Under ketamine/metedomedine anesthesia, 2-3×10^4 cells were stereotactically transplanted into the striatum of each animal (coordinates: AP +0.9 mm, ML −2.6 mm and V −4.0 mm; toothbar set at 0.0); control rats received a similar injection with mouse embryonic fibroblasts (MEF). Rotation behavior was evaluated 4 and 8 weeks post grafting. Daily *s.c.* injections of cyclosporine A 15 mg/kg (Sigma-Aldrich) were given, starting 24 hrs before cell grafting (double dosage), and continued until the rats were sacrificed and perfusion-fixated at 9 weeks after cell grafting.

All animal experiments were carried out according to the Dutch Regulations for Animal Welfare. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Groningen.

Tissue fixation and histology

Under ketamine/metedomedine anesthesia, rats were transcardially perfused with 4% paraformaldehyde (PFA) in PBS. Brains were explanted, post-fixed in 4% PFA for 24 hours and soaked in a 20% sucrose solution for 1 day. They were sectioned (14 µm sections) on a cryostat after embedding in O.C.T. compound (Sakura Finetek, Torrance, USA).

Statistical analysis

All tests were performed and analyzed in a blinded manner. Throughout the text and in the figures, all values are expressed as the mean ± SEM. SPSS analysis of difference in means between 2 groups of independent samples were analyzed using student's t test. Differences in means among multiple data sets were analyzed with one-way ANOVA by Tukey post-hoc analysis. In all analyses, P values of less than 0.05 were considered significant.

RESULTS

Dopaminergic neuron differentiation of iPSc lines

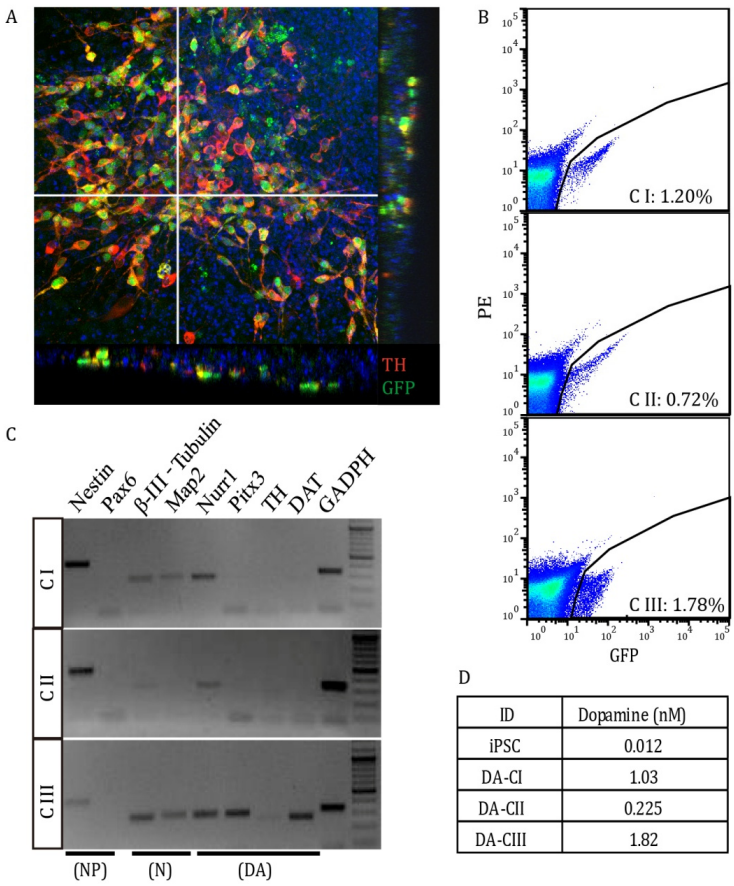
iPS cells were generated from embryonic fibroblasts isolated from Pitx3$^{gfp/+}$ transgenic mice. The lineage-specific differentiation of three iPSC clones into VM DA neurons was performed according to previously described protocol [18]. The appearance of Pitx3-expressing DA neurons in the differentiating iPS cells could be detected by their expression of GFP; at day 20 of differentiation most of the Pitx3-GFP+ cells co-expressed tyrosine hydroxylase (TH) (Figure 1A). The green fluorescent protein (GFP) expression under control of the Ptix3 promoter allowed purification of the differentiated DA neurons with FAC-sorting [21]. Of the three iPSC clones used, clone III showed the highest yield of GFP+ cells (1.78%) after sorting (Figure 1B). Of all 3 sorted Pitx3 GFP+ cell suspensions, mRNA was collected and analyzed with RT-PCR. Only the purified Pitx3-GFP+ cells of CIII appeared to express the proper profile of DA neuron markers, i.e. both neuronal markers (Map2 and β-III-tubulin) and VM DA neuron markers (Nurr1, Pitx3, TH and DAT) (Figure 1C); apparently, despite strong immunostaining (see Figure 1A), TH mRNA expression appeared low in this stage of maturity of the DA neurons. Measurement of KCl stimulated secretion of dopamine revealed a much higher dopamine release in the CIII-derived Pitx3 GFP+ cells than those from the other 2 iPSC clones (Figure 1D). In all our following experiments, we only used the Pitx3 GFP+ DA neurons derived from iPSC clone III.

Over-expression of L1CAM and PSA-NCAM in iPS cell-derived DA neurons

The sorted Pitx3-GFP+ DA neurons were transduced with a lentiviral vector containing the genes encoding for human L1CAM or STX; an empty vector was used as control. The transduced cells were thoroughly washed before they were plated onto primary astrocyte layers. The expression of L1CAM and STX was analyzed using q-PCR. There was basal expression of mouse L1CAM and mouse STX in DA neurons transduced with control vectors; and the level of endogenous L1CAM and STX expression in astrocytes appeared to be very low (Figure 2A, C). One week post transduction, specific expression of human L1CAM mRNA could be detected at a 4 times higher level in the L1CAM vector transduced group (Figure 2A). L1CAM was also detected by immunofluorescent staining both in the soma and along the neurite of DA neurons in both groups (Figure 2B). The expression of human STX was about 10 times higher in the STX vector transduced group in comparison to the control vector group (Figure 2C). Immunostaining showed the presence of PSA-NCAM both in the soma and the neurite of DA neurons in both groups (Figure 2D). Both L1CAM and PSA-NCAM were not detectable in the primary astrocyte layer (Figure 2B, D).

L1CAM and PSA-NCAM over-expression stimulates neurite outgrowth of iPS cell-derived DA neurons *in-vitro*

Transduced DA neurons were plated onto a primary astrocyte layer to create an optimal supporting environment for outgrowing neurons enabling a clear analysis of the effects of L1CAM and PSA-NCAM over-expression on cell morphology. DA neurons co-cultured on top



ID	Dopamine (nM)
iPSC	0.012
DA-CI	1.03
DA-CII	0.225
DA-CIII	1.82

Figure 1. Dopaminergic differentiation of 3 Pitx3$^{gfp/+}$ iPSC clones
(A) The appearance of Pitx3-expressing DA neurons in the differentiating iPS cells could be detected by their expression of GFP; at day 20 of differentiation most of the Pitx3-GFP+ cells co-expressed TH as demonstrated with immunostaining. (B) Three clones (CI, CII, CIII) of iPS cells reprogrammed from mouse embryonic Pitx3$^{gfp/+}$ fibroblasts were differentiated into VM DA neurons. At day 20 of differentiation, the Pitx3-GFP+ cells of each iPSC clone were sorted. (C) mRNA expression for neural precursor (NP) markers, neuronal (N) markers and VM DA neuron (DA) markers was analysed using RT-PCR. (D) Secretion of dopamine by the 3 Pitx3-GFP+ cell cultures upon KCl stimulation was measured with HPLC. It is clear that the Pitx3-GFP+ cells differentiated from iPSC clone III, best fulfilled the criteria of DA neurons.

of a primary astrocyte layer had an oval or trigonal soma of approximately similar sizes (Figure 3A). Tracings made of DA neurons from the control group and the L1CAM over-expressing group revealed bipolar or tripolar neurite formation, while DA neurons in the PSA-NCAM group occasionally also had 5 or 6 primary neurites (Figure 3B). However, this

difference was not significant among the three groups. The morphology of the neurites in both L1CAM and PSA-NCAM groups was much more complex (Figure 3A), with more branches than in the control group (Figure 3C). The longest neurite from each TH+ DA neuron was also measured. Both L1CAM and PSA-NCAM over-expressing cells extended their neurite significantly longer than the control ones (Figure 3 D). With longer neurites and more branches, the total neurite length in these two groups was significantly longer (Figure 3 E) and covered a larger area (Figure 3 F).

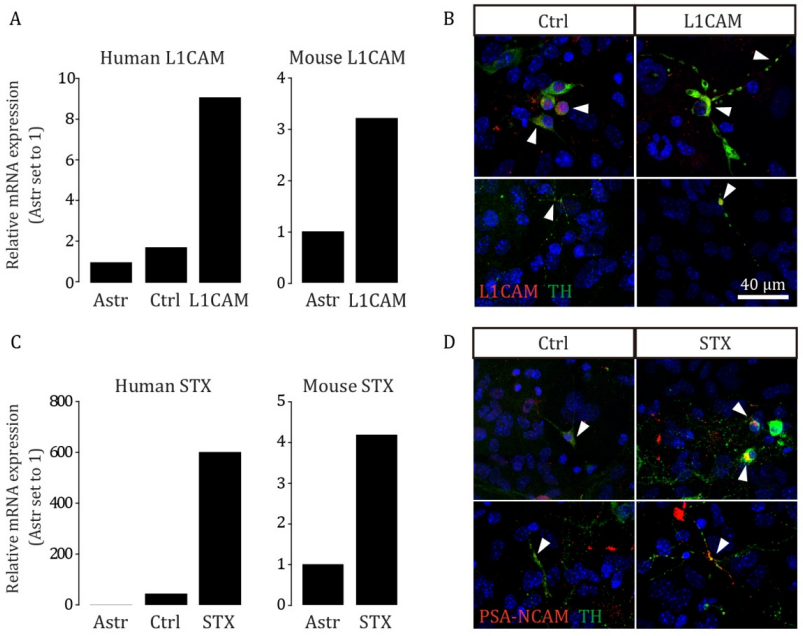


Figure 2. Over-expression of hL1CAM and hSTX in iPS cell-derived DA neurons
(A, C) Transduction of the iPS cell-derived DA neurons with hL1CAM, hSTX or control (empty) vectors resulted, after culturing for 1 week on an astrocyte cell layer, in the over-expression of hL1CAM and hSTX. The expression of human and mouse L1CAM or human and mouse STX mRNA was analyzed by q-PCR. (B, D) Expression of L1CAM and PSA-NCAM was verified by co-immunostaining of L1CAM and PSA-NCAM with TH.

L1CAM and PSA-NCAM over-expression does not promote neurite outgrowth of iPS cell-derived DA neurons in organotypic slices

To provide an environment mimicking the striatal-*in-vivo* situation, transduced iPS cell-derived DA neurons from all three groups (L1CAM or PSA-NCAM over-expressing and controls) were placed onto mouse organotypic striatal slices and cultured for 1 week. TH+ DA neurons were either clustered as groups or scattered on the slice (Figure 4A). Analysis of the number of primary neurites revealed no differences between the three groups (Figure

4C). In general, all DA neurons on the striatal slices grew much longer neurites than those cultured on the astrocyte layers (Figure 4B *v.s.* Figure 3D). Due to the neuronal clustering and the severe intertwining of the neurites, it was difficult to trace all branches from one neuron. Therefore, we only traced the longest neurite distinguishable from each neuron. No significant differences were observed in the longest neurite length between the DA neurons over-expressing L1CAM or PSA-NCAM or the control group (Figure 4B). This may be due to the high level of cell adhesion molecules such as L1CAM and PSA-NCAM already present in postnatal organotypic striatal slices (Supplementary Figure 1), already promoting maximal outgrowth of neurites from DA neurons. Remarkably, clustered DA neurons of the L1CAM and PSA-NCAM groups tended to extend their neurites in a parallel direction (Figure 4A), unlike those of the control group (Figure 4A). This phenomenon may point to the fact that L1CAM and PSA-NCAM-over-expressing DA neurons are more responsive to patterning cues in the striatal slice.

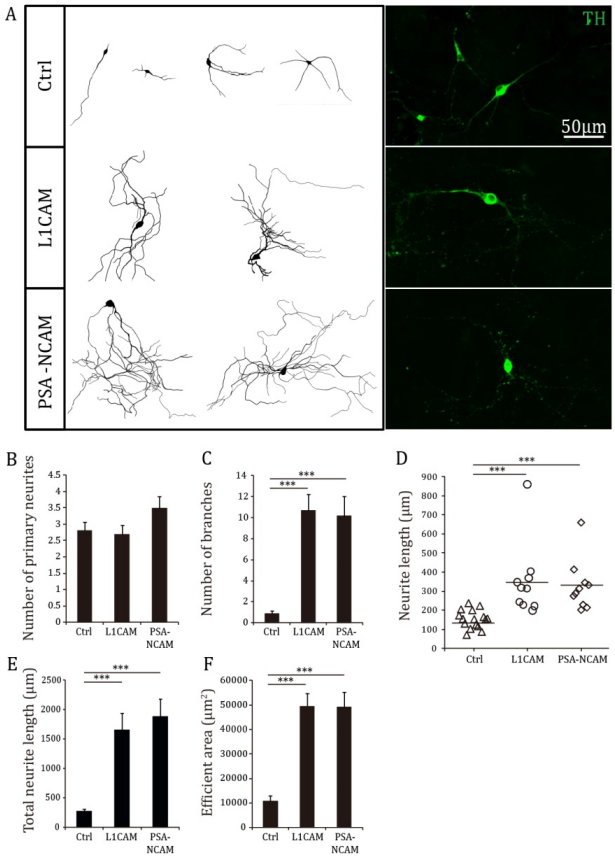


Figure 3. Over-expression of L1CAM and STX stimulated the outgrowth of iPS cell-derived DA neurons on an astrocyte layer
(A) iPS cell-derived DA neurons over-expressing L1CAM or STX were cultured on a primary astrocyte layer for 1 week. Immunostaining for TH shows the morphology of DA neurons from the three groups (including controls). Tracings of exemplary neurons are shown. The tracings were used to determine (B) the number of primary neurites, (C) the number of branches, (D) the length of the longest neurite per neuron, (E) the total neurite length and (F) the effective area (area linked-up by the far end of neurites from one neuron).. Data are presented as mean ± SEM, n = 10 to 15 per group, * $P < 0.05$, ** $P < 0.001$, *** $P = 0.000$, one-way ANOVA, Tukey post hoc analysis.

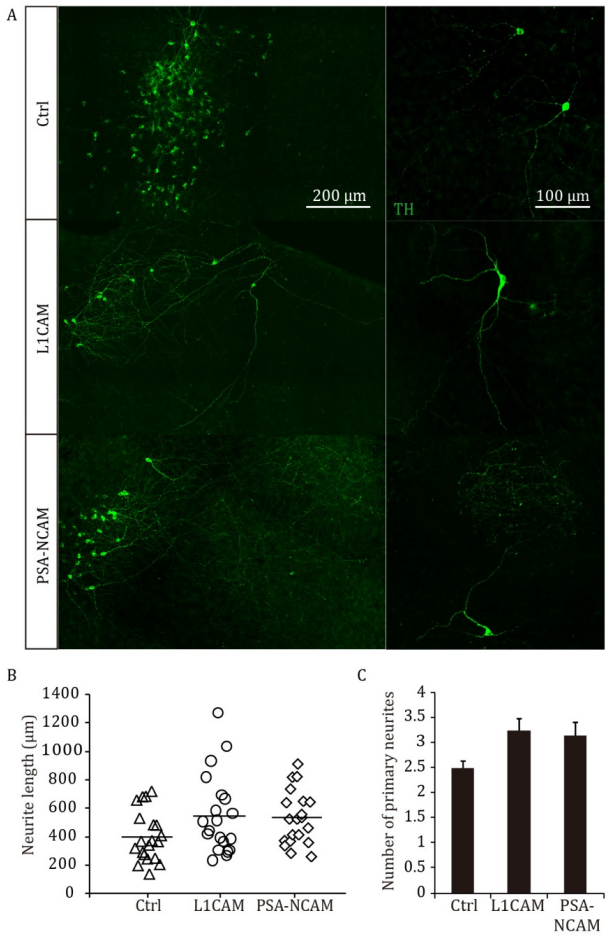


Figure 4. Outgrowth of iPS cell-derived DA neurons on organotypic striatal slices
(A) iPS cell-derived DA neurons over-expressing L1CAM or PSA-NCAM or control ones were cultured on organotypic striatal slices from P0-P3 mice. Soma and neurites of DA neurons were stained with TH. (B) The longest neurite from scattered DA neurons or cells on the edge of clustered DA neurons were measured. (C) The number of primary neurites per cell was counted. Data were presented as mean ± SEM, n = 15 per group.

Implantation of iPS cell-derived DA neurons in 6-OHDA lesion rats

Sorted control iPS cell-derived DA neurons and iPS cell-derived DA neurons over-expressing L1CAM or PSA-NCAM were transplanted into unilaterally 6-OHDA lesion rats, according to the procedure described before. Two weeks post 6-OHDA injection, rats that rotated more than 5 rpm after amphetamine injection were included in the experiments. At 8 weeks after implantation, a small reduction in the number of rotations was observed in the groups (Figure 5A). Immunohistochemical examination of the implanted striata revealed the survival of only, on average, 423 (± 108) implanted TH+ neurons in the denervated striatum. The presence of only a very small number of DA neurons may account for the small reduction in rotation behavior. Apparently, the subsequent stressful procedures of FAC-sorting and viral transduction had made the DA neurons vulnerable for the stress associated with the

implantation procedure, resulting in the survival of only about 2% of the injected cells. Accurate analysis of the 3D outgrowth of the newly-formed neurites extending from individual implanted neurons appeared to be difficult, but the maximal length of the neurites in each of the groups of implanted iPS cell-derived DA neurons did not exceed the 500 µm (Figure 5B).

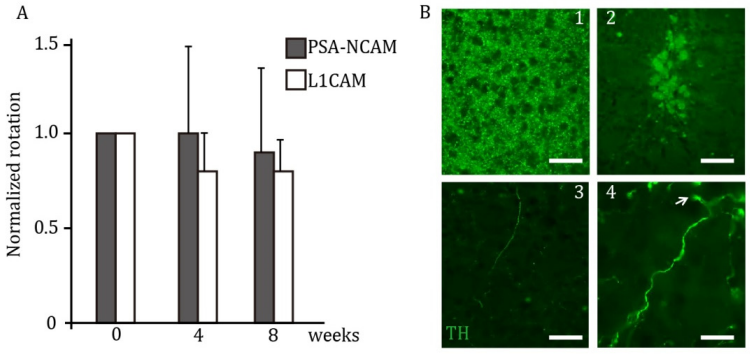


Figure 5. Intrastriatal transplantation of iPS cell-derived DA neurons in unilaterally 6-OHDA-lesioned rats

(A) Amphetamine-induced rotation of the 6-OHDA-lesioned rats transplanted with the iPS cell-derived DA neurons at 4 or 8 weeks after grafting showed a small nonsignificant reduction after 8 weeks; mean ± SEM, n = 3. (B) Immunohistochemical staining for TH shows the intact contralateral side of the unilaterally lesioned rat striatum with the punctuate dopaminergic terminals (1), which is completely gone at the ipsilateral side after dopaminergic denervation by 6-OHDA lesioning of the SN DA neurons (2); in this denervated striatum a cluster of TH+ implanted neurons can be detected at the side of implantation. Individual neuronal cell bodies (arrow) from these clusters extend their thin, curly neurites in the striatum (3,4). Bar = 100 µm (1 & 3) or 50 µm (2 & 4).

DISCUSSION

Considering the option of a cell replacement therapy for Parkinson's disease, iPS cells are presently the most promising source for an unlimited number of autologous DA neurons to be grafted intracerebrally. The *in-vitro* differentiated, iPS cell-derived DA neurons have been demonstrated to adopt the characteristics of true (ventral mesencephalic) DA neurons as assessed by cell type specific marker expression, electrophysiology and dopamine release [11, 22]. However, comparison of the genome wide expression profile of iPS cell-derived DA neurons to primary DA neurons, showed a subset of genes that were down-regulated in iPS cell-derived VM DA neurons. Gene annotation analysis showed that these genes were involved in nervous system development, neuron differentiation, and neurogenesis [21]. This may explain the fact that iPS cell-derived DA neurons are not as efficient as primary DA neurons in reinnervating the DA depleted striatum in Parkinson 6-OHDA rats and giving rise to functional recovery (reviewed in chapter 2). Extensive striatum-wide reinnervation is an absolute pre-requisite for a DA neuron replacement therapy for PD to be successful. Yet the

short neurites extending from grafted iPS cell-derived DA neurons appeared to be restricted within a short range around the injection track [22-23]. In the present study, we attempted to improve the neurite outgrowth of iPS cell-derived DA neurons by forcing their over-expression of L1CAM and PSA-NCAM, both crucial adhesion molecules for axon guidance during embryonic neuronal development.

iPS cells were reprogrammed from fibroblasts of Pitx3$^{gfp/+}$ mice [24], and subsequently differentiated into DA neurons. The various iPSC lines used demonstrated a different efficiency in *in-vitro* DA neuron differentiation, not only as far as the yield of Pitx3-GFP positive DA neurons is concerned, but also with regard to the expression profile of VM DA neuronal markers in these Pitx3-GFP positive cells and their production of dopamine. The transcription factor Pitx3 has been demonstrated to be one of the most stringent markers for fully differentiated, functional VM DA neurons [25-26]. Yet, apparently, the expression of Pitx3 in the iPS cell-derived DA neurons did not guarantee uniformity in the full differentiation of DA neurons derived from different iPSC lines. This must be related to differences in the completeness of reprogramming and the remnant of epigenetic markers interfering with complete DA neuron differentiation. These findings emphasize the importance and need for extensive detailed analysis of PD patient iPS cell-derived DA neurons, ultimately prepared for clinical usage.

Inducing over-expression of L1CAM and PSA-NCAM in DA neurons derived from iPS cells appeared to be cumbersome. Our first approach was to transduce the relevant genes in the iPS cells and to establish stably transduced iPSC lines (Supplementary Figure 2) that were subsequently differentiated into DA neurons. However, L1CAM expression was lost during differentiation and could only be detected in the undifferentiated cells (Supplementary Figure 3). This is in accordance with previous observations on transgene silencing when transduced stem cells differentiate into specialized cell types [27-28]. Moreover, the yield of Pitx3$^{+/gfp}$ DA neurons was reduced to 0.7% due to the iPSC transduction procedure employing puromycin (Supplementary Figure 3). We decided to perform lentiviral h*L1CAM* and h*STX* gene transduction on a suspension of Pitx3-GFP FAC-sorted DA neurons and were able to show stable up-regulation of both hL1CAM and hSTX in the transduced iPS cell-derived DA neurons. These L1CAM and PSA-NCAM over-expressing iPS cell-derived DA neurons allowed us to evaluate the effect of these molecules on neurite outgrowth *in-vitro*, on top of a feeder layer of astrocytes (which did not express these molecules). We demonstrated that, indeed, the neurites in both L1CAM and PSA-NCAM groups showed a much more complex, branched morphology and overall length than those in the control group. When the iPS cell-derived DA neurons were cultured on top of organotypic slices of postnatal striatum, both L1CAM and PSA-NCAM over-expressing iPS cell-derived DA neurons as well as control iPS cell-derived DA neurons equally demonstrated extensive neurite outgrowth and complex branching. As the postnatal striatal slices are very rich in L1CAM and PSA-NCAM expression, we speculate that the iPS cell-derived DA neurons were already promoted to grow their neurites to their maximum. Only, the pattern of outgrowth of the L1CAM or PSA-NCAM over-expressing iPS cell-derived DA neurons appeared slightly

different than the control ones, indicating a different response of these cells to the cues in the substrate.

We intended to examine the effect of L1CAM and PSA-NCAM on the neurite outgrowth of iPS cell-derived DA neurons in the denervated striatum of 6-OHDA lesion Parkinson rats. Unfortunately, the subsequent stressful procedures of FAC-sorting and lentiviral transduction made the DA neurons vulnerable for the stress associated with the implantation procedure, resulting in the survival of only about 2% of the injected cells. Low survival (<3%) of implanted iPS cell-derived DA neurons have been described before [29-30] and sorting before transduction has been shown to further reduce the survival rate to less than 0.06% [22]. Although our *in-vitro* experiments clearly revealed the outgrowth stimulating ability of L1CAM and PSA-NCAM, pronounced outgrowth stimulation could not be detected in the few surviving L1CAM and PSA-NCAM over-expressing iPS cell-derived DA neurons in the denervated striatum of the unilaterally 6-OHDA lesion rats. This in contrast to recent findings by Battista *et al.* [31] who demonstrated that PSA-NCAM over expression was able to enhance neurite outgrowth of stably transduced mESC-derived DA neurons *in-vivo*. Moreover, experimental studies with the spinal cord injury model clearly demonstrated that L1CAM [32] and PSA-NCAM [33] were able to enhance axon regeneration and extension. It seems likely that the sorting, the lentiviral transduction of the sorted DA neurons and the implantation protocol affected the viability and health of the surviving DA neurons annihilating the growth promoting effects of the adhesion factors.

In conclusion, we have demonstrated that L1CAM and PSA-NCAM significantly stimulate the neurite outgrowth of iPS cell-derived DA neuron, both neurite extension and branching *in-vitro*. We failed to demonstrate actual neurite outgrowth stimulation in iPS cell-derived DA neurons after implantation in the denervated striatum, but we assume that this is only due to the general poor health of the cells as a consequence of the subsequent stressful treatments of these cells before and during implantation.

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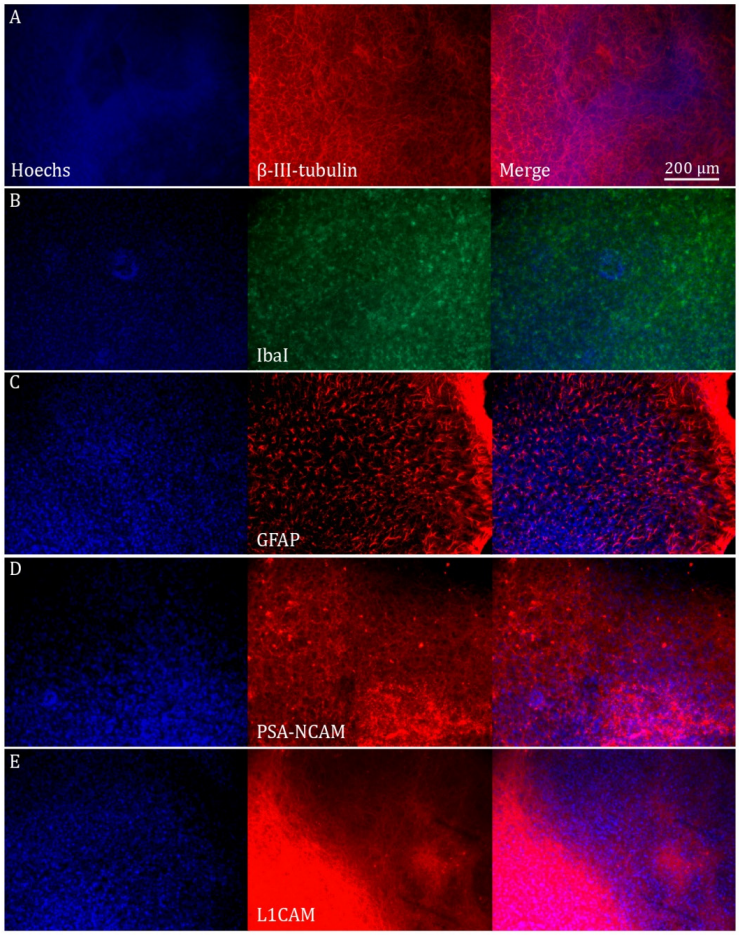
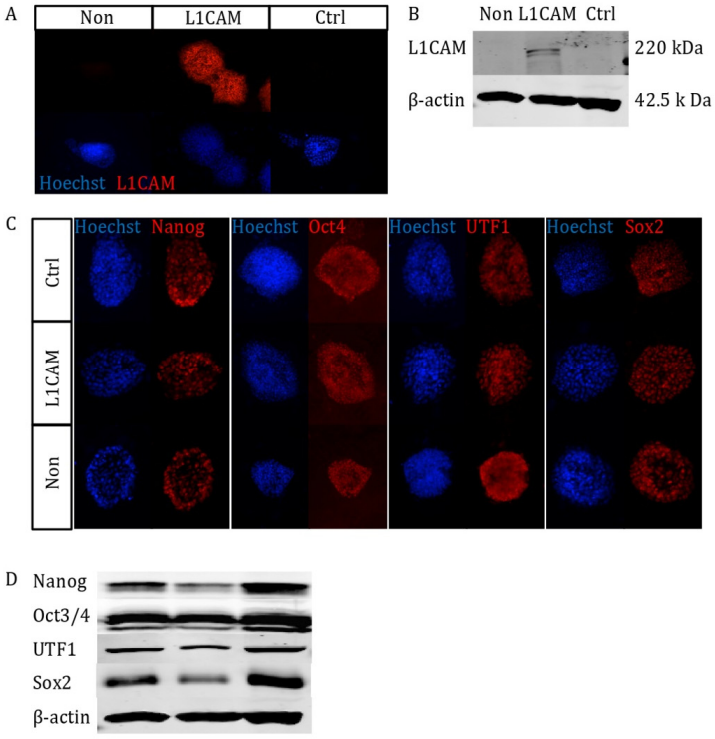
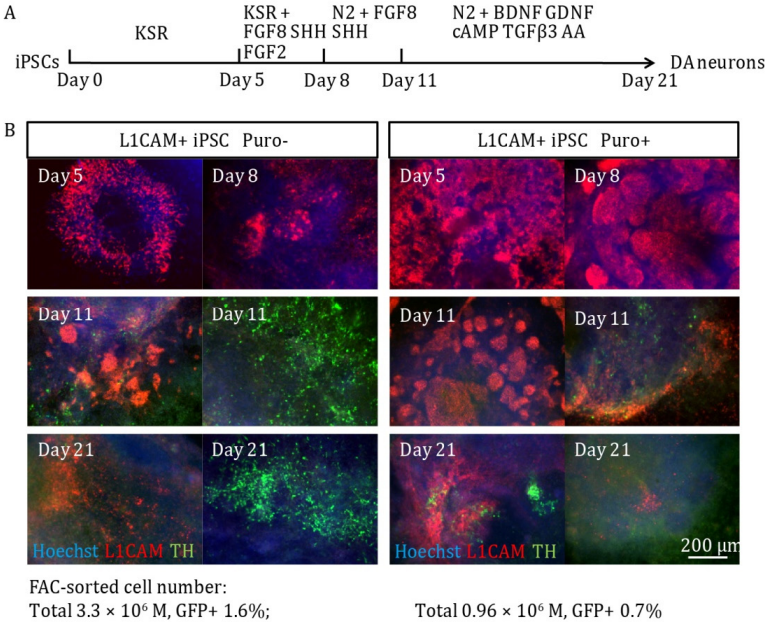


Supplementary Figure 1. Organotypic striatal slice culture
Striatal slices were cut from the striatum of P0-P3 mice with a thickness of 250 µm. The slices were cultured for 2 weeks before sorted DA neurons were layered on them. Striatal slices were characterized by immunostaining for (A) the neuronal marker β-III-tubulin, (B) the microglia marker IbaI, (C) the astrocyte marker GFAP, and (D) the cell adhesion molecules L1CAM, (E) and PSA-NCAM.



Supplementary Figure 2. Transduction of L1CAM in iPS cells
After lentiviral transduction, L1CAM was found to be stably expressed after selection by puromycin exposure for several passages. L1CAM was detected by both (A) IF staining, and (B) WB. (C, D) The pluripotent markers were detected in all three iPSC lines: non-transduced, L1CAM transduced and control factor transduced ones.



Supplementary Figure 3. Reduction of L1CAM expression in iPS cells
(A) Scheme of the DA neuron differentiation procedure. The time points of sampling for IF staining were indicated. (B) L1CAM expression was gradually reduced during differentiation. In the presence of puromycin, L1CAM expression can be much more pronounced during differentiation, yet it is mostly in the round and compact pluripotent-like cells, instead of in cells with a neural morphology. By the end of differentiation, L1CAM was mostly not co-localized in the clusters of TH+ cells.

Supplementary Table 1. Primer sequence for RT-PCR and q-PCR

Primer	Forward	Reverse
Pax6	CGGAGTTATGATACCTACACCC	GTGAAATGAGTCCTGTTGAAGTG
Nestin	AGGTGGGCAGCAACTGGCA	TCAGCCTCCAGCAGAGTCCTGT
Map-2	CTTCGGCTTATTAACCAACCA	AGTAACAATTTGTACCTGACCC
β-III-tubulin	TGTTCAAACGCATCTCGGAG	TCCATCTCATCCATGCCCTC
Nurr1	AGGGAACTGCACTTCGGCGG	GCCCGTCAGATCTCCTTGTCCG
Pitx3	CTTCCAGAGGAATCGCTACCCT	CTGCGAAGCCACCTTTGCACAG
DAT	GTTTAGGTTTAGTGGTTTTTG	TCTTAATCCTTACCTACCTCCA
TH	GAGATCGCCTTCCAGTACAG	TGGTGTAGACCTCCTTCCAG
GADPH	CATCAAGAAGGTGGTGAAGC	ACCACCCTGTTGCTGTAG
human L1CAM	TACAACGTCACCTACTGGCG	AGGATCACGGAGGTGGTGTT
mouse L1CAM	CCTGCCTGCTCATACAGATTC	CAGCGGAACTCCACTTGGG
human ST8SIA2	GGCAGAGGTACAATCAGATCAGC	ATGTCTCCATTTGGACGAGGC
mouse ST8SIA2	AGGCAGAGGTACAATCAGATCA	GAGAGAGCGTCTGGTTGTGTC